

DECONTAMINATION AND DETOXIFICATION WITH SPONGES

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ABSTRACT

One of the serious problems that may be encountered while caring for personnel contaminated with organophosphate (OP) chemical warfare nerve agents is the possibility that there will be cross-contamination to the medical personnel. Secondly, during combat or terrorist acts, individuals might be exposed to chemical toxins before they don their protective gear. Therefore, we have attempted to develop an enzyme immobilized polyurethane foam which can effectively decontaminate the skin and other such exposed surfaces of the organophosphate toxins. Antidotal therapy using cholinesterases (ChE) to scavenge the toxicity caused by OP chemical toxins is an effective parenteral pretreatment in animals against a variety of OP compounds. To continuously detoxify OPs, the ChE is combined with an oxime so that the catalytic activity of OP-inhibited ChE is continuously restored. In addition to this *in vivo* antidotal therapy, the *in vitro* reactivation of OP-inhibited ChEs by oximes also has important applications for the decontamination of skin. We have demonstrated the rapid *in-situ* copolymerization of ChEs at room temperature, and that ChE-sponges exhibit high activity and stability. In addition to the decontamination of skin and personnel, the enzyme-sponges can be utilized for preventing cross-contamination of medical and clinical personnel. The source of OP contaminants in the environment could be enclosed and detoxified if the ChE-sponge were incorporated into firefighting foams. Indeed, the sponge should be suitable for a variety of detoxification and decontamination schemes for both chemical weapons and civilians exposed to pesticides or highly toxic OPs such as sarin, or for first responders who could be exposed to OPs resulting from a terrorist act.

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INTRODUCTION

It was previously demonstrated that a variety of enzymes exhibited enhanced mechanical and chemical stability when immobilized on a solid support, thus producing a biocatalyst. The study of degradation of organophosphates by immobilized enzymes dates back to Munnecke (1), who attempted to immobilize a pesticide detoxification extract from bacteria by absorption on glass beads. The absorbed extract retained activity for a full day. Wood and coworkers (2), using isocyanate-based polyurethane foams (Hypol®), found that a number of different enzymes could be covalently bound to this polymer and retain their activity; after that Havens and Rase (3) successfully immobilized parathion hydrolase.

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More recently, the enzyme bioscavenger approach (4, 5) has been shown to be effective against a variety of OP compounds *in vitro* and *in vivo*; pretreatment of rhesus monkeys with fetal bovine serum (FBS) acetylcholinesterase (AChE) or equine serum butyrylcholinesterase (BChE) protected them against a challenge of up to 5 LD₅₀ of soman. While the use of cholinesterase (ChE) as a single pretreatment drug for OP toxicity provided complete protection, a stoichiometric amount of enzyme was required to neutralize the OP *in vivo*.



Figure 1. Apparatus for mixing enzymes in aqueous buffer and prepolymer.

To increase the OP/enzyme stoichiometry, enzyme pretreatment was combined with oximes such as HI-6 so that the catalytic activity of OP-inhibited AChE is rapidly and continuously restored before irreversible aging of the enzyme-OP complex can occur. Thus, the OP is continuously detoxified. Based on the two above observations, (a) that polyurethane foams are excellent adsorption materials for OPs (6), and (b) that soluble ChEs and oxime together have the ability to detoxify OP compounds, we combined these components in a porous polyurethane foam formed *in situ* from water-miscible hydrophilic urethane prepolymers and the enzymes. Thus, we envision a reusable immobilized enzyme sponge of cholinesterases and oximes for OP decontamination.

METHODS

SPONGE SYNTHESIS AND ASSAY.

The immobilized enzyme-sponge can be synthesized and cured in less than 20 minutes at ambient temperature and molded into the shape of any container (7). A new technique (8) was utilized to mix the prepolymer (Hypol prepolymer TDI 3000, Hampshire Chemical, Lexington, MA) and enzyme in buffer containing 1% surfactant (Pluronic P-65, BASF Specialty Chemical, Parsippany, NJ). This method replaces the rapid mixing by an electric drill with a mixing stator (a stationary plastic disposable tube for two-component mixing) to effectively reduce high shear stress and partial denaturation of the enzymes during mixing of the two components, prepolymer and enzyme (Figure 1). In addition to simplicity and easy scale-up, the activity of the ChEs coupled to the prepolymer increased by using the mixing stator compared to the high-speed drill mixing (data not shown). Depending on additives to the prepolymer, different size and length mixing stators were used to promote effective mixing. The decontamination sponge containing the immobilized enzymes were molded in a Tupperware® container to the size of a human hand as is shown in figure 2. A modified Ellman method was used to determine the effects of temperature, environment, inhibition by OPs, and reactivation by oximes on the ChE activity of the sponges (4,8).



Figure 2. Top sponge, activated carbon incorporated during synthesis; bottom sponge, immobilized AChE sponge.

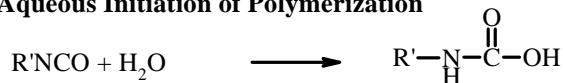
BACK-TITRATION MONITORING OF SPONGE DECONTAMINATION OF GUINEA PIG SKIN.

After the guinea pig skin was wiped with the sponge(s), each sponge was placed in a separate 50 mL capped polypropylene tube and thoroughly mixed by vortexing. Then, an aliquot was removed and placed in 1 mL

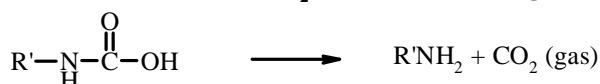
tubes containing 0.05% bovine serum albumin and 50 mM potassium phosphate buffer pH 8. The samples were sequentially diluted in the same buffer. Aliquots of all the dilutions were next transferred to a 96-well microtiter plate containing acetylcholinesterase (typically, 0.055 units). After incubation of the diluted soman with the known quantity of cholinesterase, 10 μ L was removed to a second 96-well

(a)

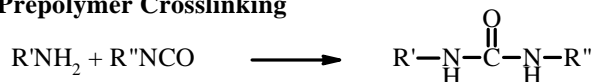
1. Aqueous Initiation of Polymerization



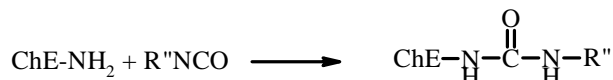
2. Amine Formation and CO₂ Evolution (foaming)



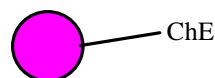
3. Prepolymer Crosslinking



4. Covalent ChE Incorporation at Aliphatic Amino Group(s)



(b)



(c)

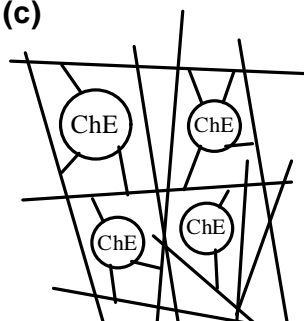


Figure 3. (a) Reactions 1-4 show the interaction of the prepolymer with water and free amino groups on the surface of cholinesterases (ChEs) or any protein. (b) Classical covalent linkage to a preformed solid support is shown in the upper right, where the enzyme is distant from the support. (c) In contrast, the result of *in situ* polymerization with enzyme is depicted in the lower right, where the enzyme becomes cross-linked to and a part of the matrix during synthesis. In this manner, the enzyme gains some of the structural integrity of the cross-linked polymer, including resistance to environmental denaturing conditions.

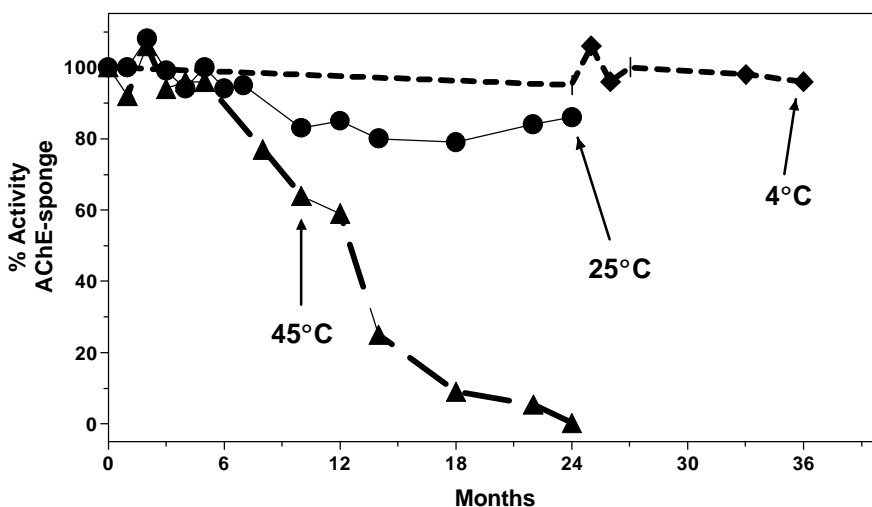


Figure 4. Immobilized AChE after continuous exposure to the indicated temperatures.

microtiter plate and inhibition determined using a Molecular Devices Plate Reader and a modified Ellman procedure as previously described (4,8). In this manner, the soman samples were diluted between 10^5 and 10^{11} fold, permitting quantification of the resulting inhibition of the cholinesterase (10-90% activity remaining) in at least one of the dilutions.

GUINEA PIG DECONTAMINATION.

Animal use: The protocols for the animal experiments were approved by the U.S. Army Medical Research Institute of Chemical Defense Committee on Animal Care and Use, and research was conducted in compliance with the Animal Welfare Act and other Federal statutes and regulations relating to animals and experiments involving animals, and adheres to principles stated in the Guide for the Care and Use of Laboratory Animals, National Research Council, NRC publication 96-23, 1996 edition.

M291 decontamination kit: The sedated and shaved guinea pigs were cutaneously exposed to neat soman on their sides. One minute after the exposure, an M291 pad, previously removed from the M291 kit and cut it in half, was held in forceps and the guinea pig was decontaminated using five counter clockwise swipes. The second half of the pad was used to perform an additional five clockwise swipes.

Sponge: The sedated and shaved guinea pigs were cutaneously exposed to neat soman on their sides. One minute after the exposure, a sponge wrapped around a pair of forceps was moved across the guinea pig's side; then the forceps were rotated 180 degrees, so that the clean surface of the sponge was pointed at the animal. Three more passes were taken from the rear towards the front. An identical procedure was used when the protocol required an additional second sponge to decontaminate the animal.

RESULTS

Previously, we described the development of a product, composed of cholinesterases (ChEs), oxime, and polyurethane foam (PUF) combinations, for the decontamination of organophosphorus compounds (OPs) from sensitive biological surfaces such as skin. Fetal bovine serum-AChE and equine-

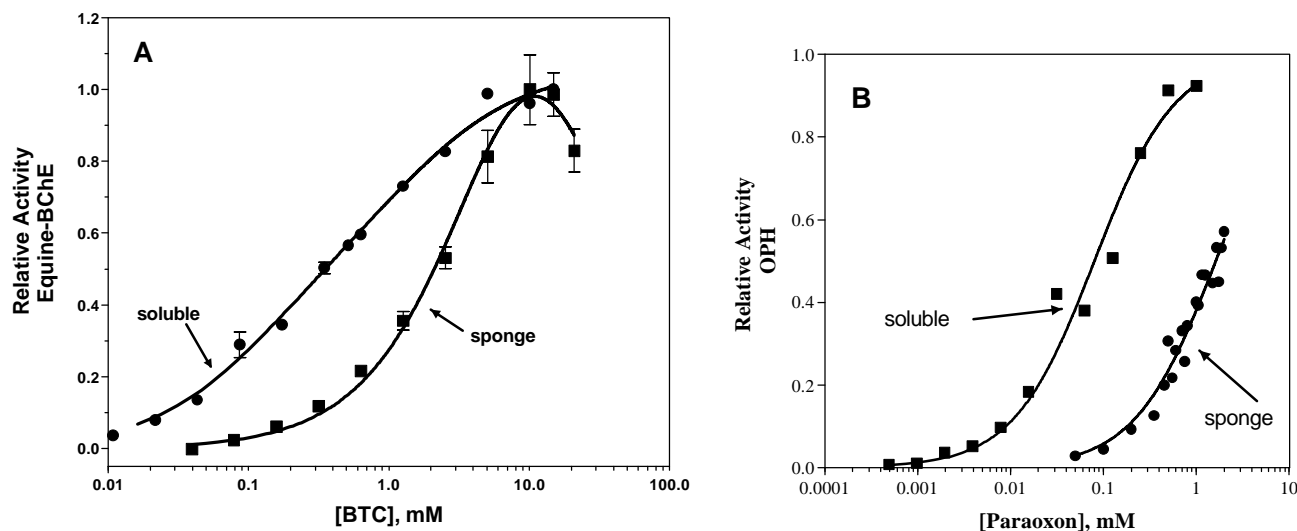


Figure 5. Substrate and activity curves for soluble and immobilized BChE and OPH. Note the shift to right for the immobilized enzymes (BChE and OPH) in the sponge compared to the soluble form of the enzymes.

BChE, which were purified using procainamide-Sepharose 4B affinity chromatography to apparent homogeneity, were immobilized by covalently linking the enzymes to polyurethane foams yielding a product having the consistency of sponges. We have demonstrated that the ChEs are covalently linked to the polyurethane matrix since immobilized ChEs showed little leakage from the PUF matrix. This linkage has the added benefit of enhancing the thermal stability of the ChEs to elevated temperatures (7). We now report further studies of the immobilized enzymes and characterization of the polyurethane foams.

Our recent results now demonstrate the following characteristics of sponges containing immobilized AChE. We evaluated different polymers for immobilization of the enzymes. Originally, we used tolyl diisocyanate (TDI, 5%, scheme shown in Figure 3) and methylenedi-phenyl diisocyanate (MDI, 5%) polyether prepolymers. The TDI prepolymer proved more suitable to enzyme immobilization, presumably due to its flexible structure, and the TDI yielded ChE-sponges with enhanced resistance to environmental denaturation. We have now evaluated prepolymers containing 3% TDI and 5% isophorone diisocyanate. The latter prepolymers yielded about the same efficiency for covalently coupling AChE and retained a high degree of esterase activity. The TDI polyurethane and enzyme matrix results in a ChE sponge of remarkable enzymatic stability. Specifically, ChE-sponges retained their original activity after 3 years at 0°C and after 2 years at 25°C, and more than 50% activity after more than 6 months at 45°C (Figure 4).

The K_m values for immobilized BChE and immobilized OPH (Figure 5A and 5B, respectively) were about 10-fold greater than for the corresponding soluble enzymes, as demonstrated by the rightward shift of the substrate (in mM) vs. activity curves. Note that while immobilized BChE yielded substrate inhibition, in contrast, the soluble form of BChE lacked substrate inhibition. These observations suggest that covalent binding of the polymer to surface residues of ChEs and OPHs caused changes to be transmitted to the active site region of the bound enzymes. On the other hand, there were no significant shifts in the pH profiles of either OPH or BChE enzymes, and the bimolecular rate constants for the inhibition of AChE and BChE in soluble or immobilized forms of the enzymes remained unchanged (8). Therefore, OPs interacted similarly with soluble and immobilized ChEs.

We also found that the OPs diisopropylfluorophosphate (DPF) or MEPQ (7-(methoxyphosphinyloxy)-1-methylquinolinium iodide) inhibited the activity of ChE-sponges, as was observed for non-immobilized ChE in solution. The oxime HI-6 restored activity of the AChE-sponge until the molar concentration of MEPQ reached approximately 1000 times that of the cholinesterase active site. However, the AChE-sponge could be recycled many times by rinsing the sponge with HI-6 in the absence of OP. In this case, most of the original ChE activity could then be restored to the sponge. Therefore, the bioscavenger approach (Figure 6) can be used externally: the sponge would soak up organophosphate decontaminating the OP contaminated skin. Then the ChE sponge and oxime would detoxify the OP in the sponge. We have found that the ability of the immobilized enzymes and HI-6 to detoxify the OP MEPQ was dependent upon the efficiency of the sponge to decontaminate particular surfaces.

Experiments were performed with sponges lacking enzyme so that we could directly evaluate the ability of the sponge to decontaminate the skin. The sponge alone could remove and decontaminate more than 97% of the MEPQ from non-porous plastic and stainless steel surfaces (Figure 7), and an AChE-sponge with HI-6 detoxified the removed MEPQ. However, the sponge without enzyme was not more effective than the M291 decontamination kit for removing neat soman (GD) applied to guinea pig skin (figure 8). We therefore evaluated additives to the polyurethane matrix, both during synthesis and post synthesis, to improve the removal and extraction of OPs from guinea pig skin.

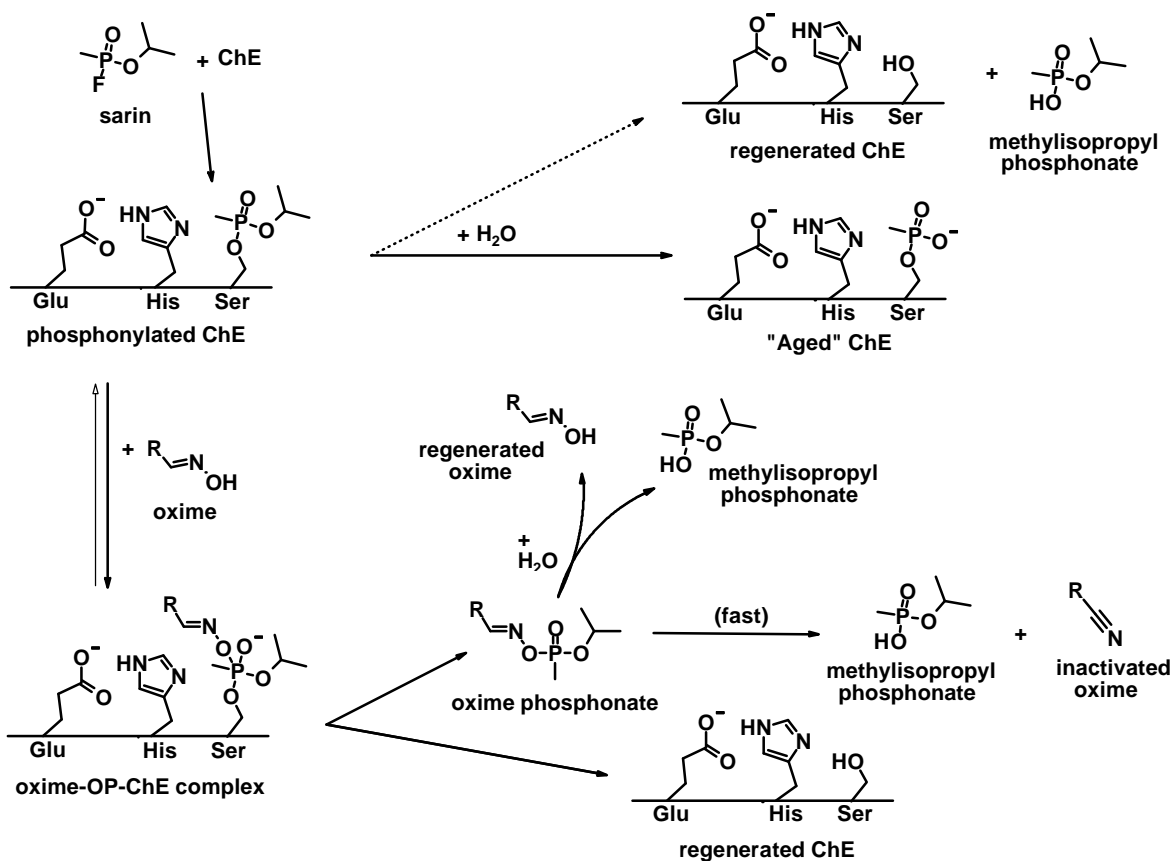


Figure 6. Inhibition of soluble or immobilized cholinesterase by organophosphate (sarin) and reactivation of the alkylphosphonylated enzyme by oxime.

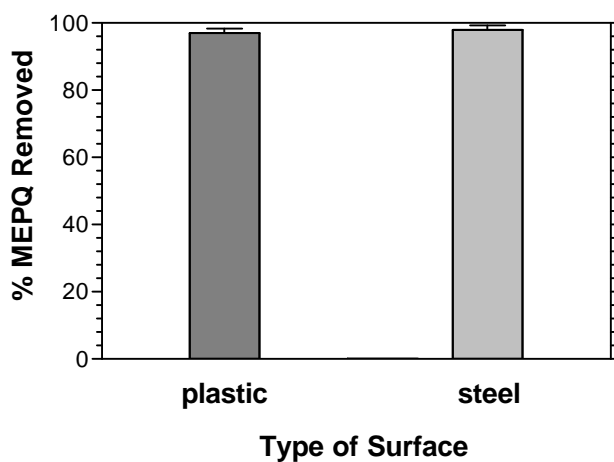


Figure 7. Removal of the organophosphate MEPQ from solid surfaces.

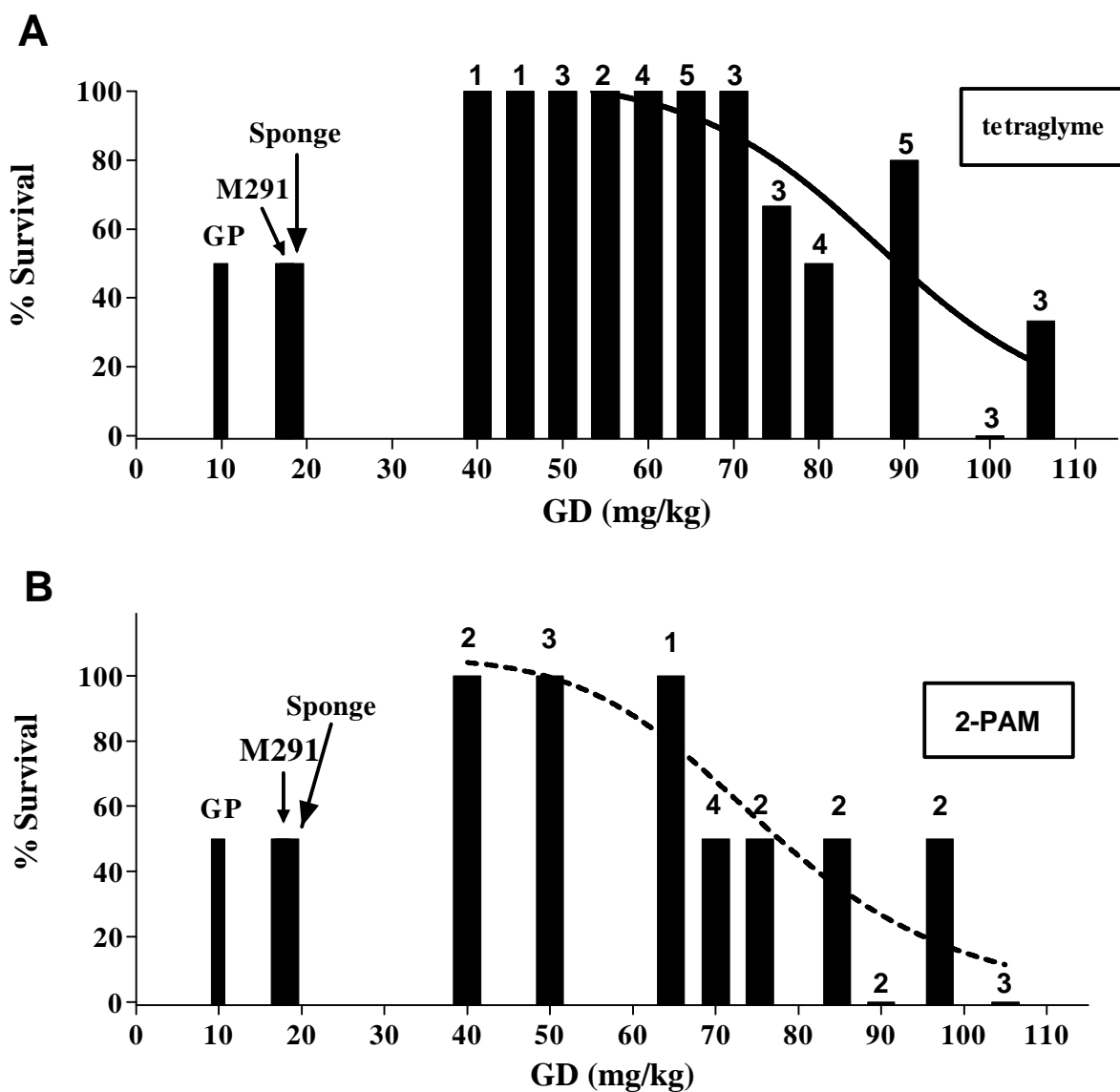


Figure 8. Detoxification of neat soman on guinea pig skin by the M291 decontamination kit, sponge without enzyme or additives, and sponge with additives. The values above the bars indicate the number of animals tested.

We were unable to modify the prepolymer since currently there is no formulation of prepolymer with an increased hydrophobic nature that might be expected to absorb the OP more effectively. Liquid additives possessing surfactant properties, zwitterion and buffers, and partial organic solubilizing characteristics were tested, including centrimide, 18-crown-6, iso-octane, kryptofix 222, polyethylene glycol 6000, triacetin, and tetraglyme. We found that most solutions provided no significant benefit over the original phosphate buffer included in the sponge that was optimized for enzyme activity. However, both triacetin and tetraglyme (Figure 8A) provided additional ability to remove soman from the skin, protecting guinea pigs about four to five-fold better than the M291 kit. In addition, sponges were synthesized so that activated carbon would be incorporated into the polymer matrix. The addition of carbon (Figure 2) did not interfere with the immobilization of ChEs. Sponges containing the oxime 2-PAM also showed increased protection to soman skin toxicity compared to the M291 kit, at least four-fold (Figure 8B and Table 1).

The capacity of sponges to remove GD from guinea pig skin was determined using the back-titration method (see *Methods* section). The line labelled Tube shows the validation of this technique (figure 9). The slope of this line is in effect 1 and it extrapolates through the origin (where $x = 0$, $y = 0$), demonstrating that the mg of GD added to the Tube yielded a 1:1 ratio to the amount of GD found in the titration assay. The line depicting the amount of GD removed by sponge containing tetraglyme (Sponge-TG) exhibited a shallower slope (0.66), indicating that this combination did not remove all the GD from guinea pig skin. Sponge composed of only activated carbon (Sponge-carbon) was less effective than the Sponge-TG in removing soman from the animal's skin (slope = 0.55). In neither case were the sponges saturated with GD because the data did not curve at the higher amounts of GD, and both of these curves also extrapolated to the origin. In part, the inability of Sponge-TG to remove all the GD might reflect the rapid penetration of GD through skin and that tetraglyme cannot extract this fraction.

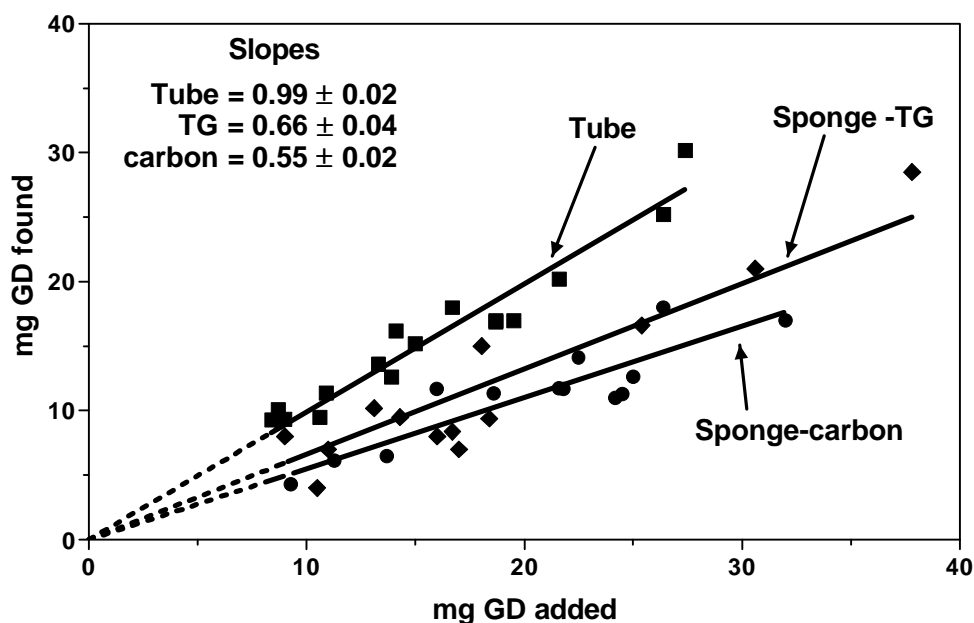


Figure 9. Determination of GD removed from guinea pig skin by different sponges based on the back-titration assay described in *Methods*: Sponge-TG, sponge with tetraglyme; Sponge-carbon, sponge synthesized with activated carbon; Tube, GD placed directly into tubes with buffer.

TABLE 1. Protective ratio of the M291 kit and sponge with additives.

Additive to sponge	LD₅₀ (mg/kg)	Protective Ratio
HI-6 (oxime)	79	8.0
2-PAM (oxime)	76	7.7
Tetraglyme	88	8.9
2-PAM + Tetraglyme	137	13.8
Reference values		
M291 decon kit	17.7	1.8
Soman alone	9.9	-

Next, we evaluated the combination of oximes with tetraglyme. In this decontamination treatment using sponges containing 2-PAM and tetraglyme, the protective ratio of the sponges over the M291 kit increased to more than 7-fold, and the LD₅₀ of soman increased to 137 mg/kg (Table 1). This compares to LD₅₀ values of 9.9 and 17.7 mg/kg for untreated animals (not decontaminated) and the M291 kit, respectively.

CONCLUSIONS

We have demonstrated the rapid *in-situ* copolymerization of ChEs at room temperature, and the ChE-sponges exhibit high activity and stability, making them suitable for a wide variety of decontamination tasks. We have evaluated decontamination schemes using the sponge alone by incorporating additives to aid in the removal of OPs from biological surfaces, rather than smooth and solid objects. Then, detoxification of the OP in the enzyme-sponge pad with oxime would take place, in so doing preventing secondary contamination. In addition, in the presence of oxime, the enzyme-sponge would be reusable. The sponges should be suitable for a variety of biological surface detoxification and decontamination schemes for both chemical weapons and pesticides directed against ChEs.

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